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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	John T. Gray, Jeng-Shin Lee and Richard C. Mulligan	
Application No.:	09/393,795	Group Art Unit: 1636
Filed:	September 10, 1999	Examiner: G. Leffers
Confirmation No.:	3301	

For: PACKAGING CELLS COMPRISING CODON-OPTIMIZED GAGPOL SEQUENCES AND LACKING LENTIVIRAL ACCESSORY PROTEINS (AS AMENDED)

CERTIFICATE OF MAILING OR TRANSMISSION

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1-20-05 Dawn M Myers
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TRANSMITTAL OF DECLARATION OF JOHN T GRAY, PH.D. AND JENG-SHIN LEE, M.D., PH.D. UNDER 37 C.F.R. § 1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Please find enclosed two executed original Declarations of John T. Gray, Ph.D. and Jeng-Shin Lee, M.D., Ph.D. under 37 C.F.R. § 1.132 for filing in the above-referenced patent application. Each Declaration is identical. One original Declaration is signed by Dr. Gray, and the other original Declaration is signed by Dr. Lee.

COPY

This Rule 132 Declaration is submitted to correct an inadvertent error in the Declaration of John T. Gray, Ph.D. filed on March 17, 2003.

In particular, in the Rule 132 Declaration of Dr. Gray filed March 17, 2003, Dr. Gray, through inadvertent oversight, incorrectly stated that the experimental work disclosed therein was conducted by John Gray or under his supervision. In fact, the experimental work had been conducted by Jeng-Shin Lee or under his supervision.

The enclosed Rule 132 Declaration of Drs. Gray and Lee correctly states that the experimental work described in the Declaration was conducted by Jeng-Shin Lee or under his supervision. The experimental work described in the Rule 132 Declaration of Drs. Gray and Lee is the same experimental work described the Rule 132 Declaration of Dr. Gray filed on March 17, 2003 with some additional technical information. Thus, the enclosed Rule 132 Declaration of Drs. Gray and Lee corrects this inadvertent error in the Rule 132 Declaration filed March 17, 2003.

Respectfully submitted,

HAMILTON, BROOK, SMITH & REYNOLDS, P.C.

By Vivien Tannoch-Magin

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DEB/HL
November 26, 2004

PATENT APPLICATION
Attorney's Docket No.: 1242.1030-002 (CMCC-693p2A)

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Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

We, John T. Gray, Ph.D., of 206 Gardenia Drive, Memphis, TN 38116, and Jeng-Shin Lee, M.D., Ph.D., of 9 Bedford Lane, Lincoln, MA 01773, declare and state that:

1. We, together with Richard C. Mulligan, are inventors of the subject matter described and claimed U.S. Application No. 09/393,795, filed September 10, 1999, and currently

entitled "Packaging Cells Comprising Codon-Optimized Gagpol Sequences and Lacking Lentiviral Accessory Proteins". U.S. Application No. 09/393,795 is hereinafter referred to as the "'795 application". Papers have been filed to correct the mistake in omitting Jeng-Shin Lee's name from the list of inventors for the '795 application.

2. The experimental work described in this declaration, and presented in the following section, clearly demonstrates that packaging cell lines can be produced as described in the application by co-transfecting mammalian host cells with a plasmid or retroviral nucleotide sequence (packaging construct) comprising a codon optimized DNA sequence which encodes lentivirus *gagpol* proteins (e.g., HIV *gagpol* proteins) but not DNA sequences encoding lentivirus or HIV accessory proteins (tat, vif, vpr, vpu, nef and rev proteins and RRE) or constitutive transport elements (CTEs). The experimental work described in this declaration in the following section was conducted by Jeng-Shin Lee or under his supervision.

3. The following is a description and discussion of the work carried out and of the results which demonstrates that packaging cell lines can be produced by co-transfecting mammalian host cells with a plasmid or retroviral nucleotide sequence (packaging construct) comprising a codon optimized DNA sequence which encodes lentivirus *gagpol* proteins (e.g., HIV *gagpol* proteins) but not DNA sequences encoding lentivirus or HIV accessory proteins (tat, vif, vpr, vpu, nef and rev proteins and RRE) or CTEs.

Experimental Protocol

Materials and Methods

The packaging construct pHDMHgpm2, which comprises a codon optimized HIV *gagpol*, is described in the subject application at page 13, line 3 to page 14, line 3. The codon optimized HIV *gagpol* sequence of the packaging construct pHDMHgpm2 is not operably linked to a RRE. The packaging construct pHDMHgpm2 does not contain sequences encoding lentivirus or HIV accessory proteins (tat, vif, vpr, vpu, nef and rev proteins and RRE) or CTEs.

The pJ6omegaPuro plasmid is described in Morgenstern, J.P. and Land, H., *Nucleic Acids Res.*, 18(4):1068 (1990). The pJ6omegaPuro plasmid encodes a selectable marker for resistance to the antibiotic puromycin (Morgenstern, J.P. and Land, H., *Nucleic Acids Res.*, 18(4):1068 (1990)). The pJ6omegaPuro plasmid does not contain DNA sequences from lentivirus or HIV, particularly sequences encoding lentivirus or HIV accessory proteins (tat, vif, vpr, vpu, nef and rev proteins and RRE) or CTEs.

The pSV2-neo plasmid, which is available from the American Type Culture Collection (ATCC), encodes a selectable marker for resistance to the antibiotic G418 (Southern, P.J. and Berg, P., *J. Mol. Appl. Genet.*, 1:327-341 (1982)). The pSV2-neo plasmid does not contain DNA sequences from lentivirus or HIV, particularly sequences encoding lentivirus or HIV accessory proteins (tat, vif, vpr, vpu, nef and rev proteins and RRE) or CTEs.

293 cells are human embryonic kidney epithelial cells transformed by sheared human Ad5 DNA (Graham, F.L. *et al.*, *J. Gen. Virol.*, 36(1):59-74 (1977)). 293 cells contain no sequences encoding envelope proteins and no sequences from lentivirus or HIV, particularly sequences encoding lentivirus or HIV accessory proteins (tat, vif, vpr, vpu, nef and rev proteins and RRE) or CTEs.

293G cells are described in Ory, D.S. *et al.*, *Proc. Natl Acad Sci. USA*, 93(21):11400-11406 (1996). 293G cells are human embryonic kidney epithelial cells which express the vesicular stomatitis virus glycoprotein G (VSV-G) envelope in an inducible fashion (Ory, D.S. *et al.*, *Proc. Natl. Acad. Sci USA*, 93(21):11400-11406 (1996)). 293G cells contain DNA sequences encoding the VSV-G envelope but no sequences from lentivirus or HIV, particularly sequences encoding lentivirus or HIV accessory proteins (tat, vif, vpr, vpu, nef and rev proteins and RRE) or CTEs.

Cells were transfected with the codon optimized HIV gagpol expressing packaging construct HDMHgpm2 and a plasmid expressing drug resistance (selectable marker plasmid) (pJ6omegaPuro or pSV2-neo) using the Effectene transfection reagent according to the manufacturer's protocol (Qiagen). Briefly, plasmid DNA (packaging construct (1 μg) and a selectable marker plasmid (0.05 μg)) were mixed with Enhancer (8 μl) and EC buffer (150 μl) and incubated for about 5 minutes. Effectene Reagent (25 μl) was then added and the mixture

was incubated for 5-10 minutes to allow Effectene-DNA complexes to form. The complexes were mixed with regular media (Dulbecco's modified eagle medium (DMEM) (GIBCO/BRL) supplemented with 10% Fetal Calf Serum (FCS) (Sigma), 50 units/ml penicillin and streptomycin (GIBCO/BRL) and added directly to the cells (1×10^6). The cells were cultured for two days in regular media (DMEM, 10% FCS, 50 units/ml penicillin and streptomycin using a humidified 37°C incubator under 5% CO_2, and then split into regular media (DMEM, 10% FCS, 50 units/ml penicillin and streptomycin) supplemented with the appropriate selection antibiotic (puromycin or G418) to select for stable clones. Antibiotic-resistant clones were amplified and screened for HIV *gagpol* gene expression to select the highest expressing clones.

To analyze the production of HIV *gagpol* gene products in newly created cell lines, 2.5×10^5 cells were seeded in 6-well cluster plates and incubated for 24 hours. Supernatant was collected and assayed for p24 antigen production, a processed product of HIV *gag*. The measurement of p24 antigen quantitates the production of gag proteins by the cells. The clones that produced a high level of p24 were further analyzed for expression of HIV reverse transcriptase (RT), a processed product of HIV pol, by using a standard HIV RT assay.

Packaging Cell Lines Expressing Codon Optimized HIV *gagpol* Gene Products

A packaging cell line expressing codon optimized HIV *gagpol* gene products was constructed by cotransfecting 293 cells (1×10^6) with the packaging construct HDMHgpm2 (1 μg) and the pJ6omegaPuro plasmid (0.05 μg) as described above. After culturing the cells for two days at 37°C and 5% CO_2, the cells were split into regular media (DMEM, 10% FCS, 50 units/ml penicillin and streptomycin) supplemented with 1 μg/ml puromycin (Sigma) to select for stable clones. Puromycin-resistant clones were amplified and screened for HIV *gagpol* gene expression to select the highest expressing clones. Sixty-two cloned cell lines expressing *gagpol* at high levels were recovered and maintained in regular media supplemented with 1 μg/ml of puromycin. The p24 antigen production from the best expressing clone was 106 ng/ml p24/250,000 cells/24 hour period.

A packaging cell line expressing codon optimized HIV *gagpol* gene products was also constructed by cotransfecting 293 cells (1×10^6) with the packaging construct HDMHgpm2

(1 μg) and the pSV2-neo plasmid (0.05 μg) as described above. After culturing the cells for two days at 37°C and 5% CO_2, stable clones were selected in regular media (DMEM, 10% FCS, 50 units/ml penicillin and streptomycin) supplemented with 500 μg/ml G418 (GIBCO/BRL). G418-resistant clones were amplified and screened for HIV *gagpol* gene expression to select the highest expressing clones. Forty-eight cloned cell lines expressing *gagpol* at high levels were reccovered. The p24 antigen production from the best expressing clone was 193 ng/ml of p24/250,000 cells/24 hour period.

These cell lines provide cellular backgrounds that various envelope genes for pseudotyping, such as amphotropic MLV envelope, can be introduced.

Packaging Cell Line Expressing Codon Optimized HIV *gagpol* Gene Products and VSV-G Envelope

A packaging cell line expressing codon optimized HIV *gagpol* gene products and the VSV-G envelope was constructed by transfecting 293G cells (1 x 10^6) with the packaging construct HDMHgpm2 (1 μg) and the pSV2-neo plasmid (0.05 μg) as described above. After culturing the cells for two days at 37°C and 5% CO_2, stable clones were selected in regular media (DMEM, 10% FCS, 50 units/ml penicillin and streptomycin) supplemented with 300 μg/ml G418, 1 μg/ml tetracycline (which represses expression of the VSV-G) and 2 μg/ml puromycin (which maintains the pre-existing VSV-G gene in the 293G cell line). G418-resistant clones were amplified and screened for HIV *gagpol* gene expression to select the highest expressing clones. Sixty cloned cell lines expressing *gagpol* at high levels were selected. The p24 antigen production from the best expressing clone was 138 ng/ml of p24/250,000 cells/24 hour period.

Results

Subsequent experiments confirmed that these packaging cell lines expressed fully functional HIV gag-pol products. In addition to expressing high levels of p24, a processed product of HIV *gag*, these cell lines also express high levels of HIV RT, a processed product of HIV *pol*. Infectious viruses can be produced with these cell lines, as described in the application,

upon introduction by transfection of the remaining components for the assembly of lentiviral particles (i.e., transfer vectors with or without an *env* expression plasmid).

Thus, the results of these experiments show that packaging cell lines can be produced as described in the application by co-transfecting mammalian host cells with a plasmid or retroviral nucleotide sequence (packaging construct) comprising a codon optimized DNA sequence which encodes lentivirus *gagpol* proteins (e.g., HIV *gagpol* proteins) but not DNA sequences encoding lentivirus or HIV accessory proteins (tat, vif, vpr, vpu, nef and rev proteins and RRE) or CTEs.

The establishment of these cell lines clearly demonstrates that packaging cell lines with high level stable expression of HIV *gagpol* gene products without the addition of viral sequences encoding lentivirus or HIV accessory proteins (tat, vif, vpr, vpu, nef and rev proteins and RRE) or constitutive transport elements (CTEs) can be produced without undue experimentation following the teachings of the '795 application. None of the plasmids or cell lines used in the experiments described herein contain the lentivirus or HIV accessory proteins tat, vif, vpr, vpu, nef and rev proteins and RRE. Additionally, no CTEs were included to facilitate *gagpol* gene expression. These results also demonstrate that codon optimization does in fact enable the high level expression of HIV *gagpol* in the absence of the aforementioned accessory proteins or viral sequences.

4. We declare that all statements made in this Declaration of our own knowledge are true and that all statements made on information and belief are believed to be true. Moreover, these statements were made with the knowledge that willful false statements and the like made by us are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

John T. Gray, Ph.D.	Jeng-Shin Lee, M.D., Ph.D.
Date	11/29/2004 Date

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Packaging Cell Lines Expressing Codon Optimized HIV *gagpol* Gene Products

A packaging cell line expressing codon optimized HIV *gagpol* gene products was constructed by cotransfecting 293 cells (1×10^6) with the packaging construct HDMHgprm2 (1 μg) and the pJ6omegaPuro plasmid (0.05 μg) as described above. After culturing the cells for two days at 37°C and 5% CO_2, the cells were split into regular media (DMEM, 10% FCS, 50 units/ml penicillin and streptomycin) supplemented with 1 μg/ml puromycin (Sigma) to select for stable clones. Puromycin-resistant clones were amplified and screened for HIV *gagpol* gene expression to select the highest expressing clones. Sixty-two cloned cell lines expressing *gagpol* at high levels were recovered and maintained in regular media supplemented with 1 μg/ml of puromycin. The p24 antigen production from the best expressing clone was 106 ng/ml p24/250,000 cells/24 hour period.

A packaging cell line expressing codon optimized HIV *gagpol* gene products was also constructed by cotransfecting 293 cells (1×10^6) with the packaging construct HDMHgprm2

(1 μg) and the pSV2-neo plasmid (0.05 μg) as described above. After culturing the cells for two days at 37°C and 5% CO_2, stable clones were selected in regular media (DMEM, 10% FCS, 50 units/ml penicillin and streptomycin) supplemented with 500 μg/ml G418 (GIBCO/BRL). G418-resistant clones were amplified and screened for HIV *gagpol* gene expression to select the highest expressing clones. Forty-eight cloned cell lines expressing *gagpol* at high levels were reccovered. The p24 antigen production from the best expressing clone was 193 ng/ml of p24/250,000 cells/24 hour period.

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upon introduction by transfection of the remaining components for the assembly of lentiviral particles (i.e., transfer vectors with or without an *env* expression plasmid).

Thus, the results of these experiments show that packaging cell lines can be produced as described in the application by co-transfecting mammalian host cells with a plasmid or retroviral nucleotide sequence (packaging construct) comprising a codon optimized DNA sequence which encodes lentivirus *gagpol* proteins (e.g., HIV *gagpol* proteins) but not DNA sequences encoding lentivirus or HIV accessory proteins (tat, vif, vpr, vpu, nef and rev proteins and RRE) or CTEs.

The establishment of these cell lines clearly demonstrates that packaging cell lines with high level stable expression of HIV *gagpol* gene products without the addition of viral sequences encoding lentivirus or HIV accessory proteins (tat, vif, vpr, vpu, nef and rev proteins and RRE) or constitutive transport elements (CTEs) can be produced without undue experimentation following the teachings of the '795 application. None of the plasmids or cell lines used in the experiments described herein contain the lentivirus or HIV accessory proteins tat, vif, vpr, vpu, nef and rev proteins and RRE Additionally, no CTEs were included to facilitate *gagpol* gene expression. These results also demonstrate that codon optimization does in fact enable the high level expression of HIV *gagpol* in the absence of the aforementioned accessory proteins or viral sequences.

4. We declare that all statements made in this Declaration of our own knowledge are true and that all statements made on information and belief are believed to be true. Moreover, these statements were made with the knowledge that willful false statements and the like made by us are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

John T. Gray, Ph.D.

November 29, 2004
Date

Jeng-Shin Lee, M.D., Ph.D.

Date